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13. ABSTRACT (Maximum 200 Words)

The ability to detect carcinoma cells in the circulation of patients with breast cancer using a circulating cancer cell test developed by Cell Works and to characterize the isolated breast cancer cells using state-of-the-art multiple markers provides the ability to determine whether micrometastases have occurred and the progressive nature of the cancer. The test was designed to analyze single cells using immunofluorescence microscopy to produce molecular profiles that may be indicative of the metastatic potential of the breast circulating cancer cells. In particular, nuclear DNA can be quantified by measuring the fluorescence of bound DAPI, a DNA-specific dye, and compare the cancer cell DNA with reference WBCs on the same slide. Simultaneous measurement of g-actin content and DNA aneuploidy at the single cell level by quantitative fluorescence image analysis may be a potential assay for breast cancer risk assessment. Other markers can be assessed concurrently by quantitative immunofluorescence, such as Ki67, p27, p53, Her-2/neu, thymidylate synthase, apoptosis, and hormone receptors (estrogen and progesterone), to further characterize the cancer cells. An ultimate goal is to commercialize a diagnostic and prognostic test to provide the patients and physicians with information that may be helpful with early metastatic cancer detection, elimination of lymph node dissection, early responsiveness of patients to hormonal therapy and/or systemic drug therapy.

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Breast Cancer, Metastasis, Circulating Cancer Cells, Cancer Detection, Cancer Cell Characterization

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FOREWORD

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___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Stephen Teskr   7/13/01
PI   Signature   Date

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5. INTRODUCTION

The focus of the research is to detect and characterize carcinoma cells in the circulation of patients with breast cancer using a circulating cancer cell test developed by Cell Works. It was critical to optimize the isolation, examination of markers, and imaging procedures of this test for breast cancer since the test was developed initially for prostate cancer. In particular, the scope of the research directly concerns the acquisition of key reagents and cells for the optimization of the methodology for aneuploidy measurements and staining of markers, such as growth/invasiveness, using breast cancer cell lines, whole blood samples, and ultimately, blood from breast cancer patients. Cell number and characterization information gathered from isolated breast circulating cancer cells in blood samples using this optimized test may be indicative of whether micrometastases are forming in the blood and, if present, the progressive nature of the cancer.

6. BODY

Since our June 2000 progress report, The Cell Works Laboratory relocated twice during the fourth quarter of 2000. Our current address is 6200 Seaforth Street, Baltimore, MD 21224; phone 410-633-8133, fax 410-633-7652. The relocation and re-establishment of the laboratory disrupted our research effort for several months. However, we have essentially completed Tasks 1, 2 & 3 of the proposal. Specific progress is outlined below.

TASK 1.   Preparation of Reagents and Acquisition of Cell Lines

      1.   Reagents

As mentioned in our previous report, total nuclear DNA content is now used as a measure of aneuploidy rather than counting specific chromosomes. In addition, we have also improved our staining procedure for detecting epithelial cells circulating in the blood using a new formulation. This new formulation consists of a new antibody cocktail containing multiple anti-cytokeratin monoclonals and combining two epithelial cell markers, viz., one set of markers targeting nine different cytokeratin peptides and the other marker targeting a tumor-associated glycoprotein. All the antibodies in the new cocktail are labeled with FITC. Our previous report listed a number of different fluorescently-labeled antibodies available at Cell Works to detect nuclear antigens, oncogenes and hormone receptors. DNAase-CY5 has been used to quantitate g-actin levels and d-UTP-FITC has been use in combination with terminal deoxynucleotidyl transferase to label apoptotic cells.

      2.   Cells

Breast cancer cell lines are available from ATCC and normal breast epithelial cells are available commercially from Clonetics. Two breast cancer cell lines, viz., T47D and MCF-7 are in culture and stored cryogenically at Cell Works.

      3.   This subtask is no longer applicable.

TASK 2.   Optimize Test With Cell Lines - All subtasks are complete.

An investigation was conducted on two breast cancer cell lines to compare two markers for cell proliferation, viz., the nuclear antigen Ki67 and thymidylate synthase (TS). MCF-7 cells and T47D cells were placed on slides, fixed with 2% paraformaldehyde and incubated simultaneously with the new epithelial cell detection antibody cocktail-FITC, anti-TS-CY5 and anti-Ki67-CY3. Three images were acquired with each field of stained cells using filter cubes that can distinguish the three fluorophores.

Figure 1 shows a pseudocolor composite image of a MCF-7 cell stained simultaneously for Ki67 and TS.   **Figure 2** shows a CY3 image and a CY5 image of the same field of T47D cells. As can be seen, all the cells are stained for both Ki67 and TS. The data are presented in **Table 1** below, which shows that over 90% of the cytokeratin-positive cells

of both cell lines were also positive for both Ki67 and TS. Thus, either the nuclear antigen Ki67, or the enzyme TS (indicator for DNA synthesis) can be used as a marker for cell proliferation.

A quantitative study was conducted to measure g-actin level and DNA content in the same cell as breast cancer biomarkers. T47D cells or MCF-7 cells were added to white blood cells, spread onto slides, fixed and then stained simultaneously with epithelial cell detection antibody cocktail-FITC, p27-CY3 and DNAase-CY5 (binds to g-actin). After mounting with DAPI-containing medium, four images of each field of cells were acquired with filter cubes to distinguish FITC, CY3, CY5 and DAPI.

Figure 3 shows a color composite image of T47D cells stained for cytokeratin (green), g-actin (red) and DNA (blue). The DAPI integrated fluorescence intensity (DNA) and CY5 integrated fluorescence intensity (g- actin) were measured in each of 40 to 50 cytokeratin-positive cells and compared to values obtained with white blood cells in the same field of view. Normal prostate cells were used as a control. The quantitative data for DNA content and g-actin levels are presented in **Table 2**. The median cancer cell DNA/diploid cell (white blood cell or wbc) DNA ratios for T47D and MCF-7 are 1.7 & 2.2, respectively, while for control cells the median ratio is 1.1 (**Exp 1**) and 1.2 (**Exp 2**). The percentage of breast cancer cells with ratios of two or greater, i.e., cells that are aneuploid with respect to DNA content, is 30% and 75% for T47D and MCF-7, respectively, while the percentage for control cells was 0 (**Exp 2**) or 7% (**Exp 1**). The percentage of the aneuploid breast cancer cells that also had cell g-actin / wbc g-actin ratios above the median value was 94% and 77% for T47D & MCF-7, respectively. Thus, the simultaneous measurement of DNA content and g-actin level appears to be good marker for breast cancer. Both breast cancer cell lines were also negative for p27, a good indicator for aggressive cells.

Apoptosis can be mediated through an endogenous signaling pathway that emanates from the cell surface receptor Fas. We have conducted a preliminary study to determine if this signaling pathway is abrogated in breast cancer cell lines. T47D and MCF-7 cells were incubated for 24 hours with 100, 500 and 1000 nanograms/ml of mouse antihuman Fas IgM activating antibody (clone CH-11). As a positive control, the cells were also incubated with 1uM staurosporine, an antibiotic that will induce apoptosis in most human cell types. Induction of apoptosis was evaluated at the single cell level using a Tunel assay to look for incorporation of dUTP-FITC into DNA strand breaks and for nuclear condensation and fragmentation in DAPI-stained nuclei by fluorescence microscopy. At 100 ng/ml, anti-Fas did not induce apoptosis in either cell line, however at the higher concentration, apoptosis was induced in T47D cells. **Figure 4** shows an image of apoptotic MCF-7cells induced by 1uM staurosporine. **Figure 5** shows an image of apoptotic T47D cells induced 1 ug/ml anti-Fas IgM. Further studies are in progress.

TASK 3.   Optimize Test Using Blood Samples

　　　1. Recovery and Characterization

　　　A. Characterization

Validation experiments were carried out at our new location using the new antibody cocktail described above in TASK 1. This new staining procedure will be very helpful in validating our automated scanning/image acquisition microscopy system since it provides for an increase in fluorescence signal, a decrease in scanning time and the potential to identify cancer cells when using a single anti-cytokeratin antibody.

　　　B. Recovery

Table 3 shows recovery data for T47D and MCF-7 breast cancer cells. Recovery for the T47D cells ranged from 66% to 82% with a mean of 71.1% (standard deviation of 7.6) and MCF-7 cells ranged from 51% to 83% with a mean of 68.6% (standard deviation of 11.8).

　　　2. Interfering Substances---No longer applicable

　　　3. Stability-----Complete

TASK 4.   Patient samples

Now that the test has been validated in our new facility with the improved antibody cocktail we plan to perform more tests on blood samples from breast cancer patients. We anticipate receiving samples in the near future.

TABLE 1

USE OF MULTIPLE MARKERS FOR DETECTION OF PROLIFERATING BREAST CANCER CELLS

	MCF-7	T47D
No. of Cytokeratin Positive Cells	54	113
No. of Ki67 Positive Cells	51 (94%)**	104 (92%)**
No. of Thymidine Synthase Positive Cells	49 (91%)**	102 (90%)**

Cells were stained simultaneously with anti-cytokeratin-FITC, anti-Ki67, and anti-thymidylate synthase-CY5.

**Number in parenthesis is percentage of cytokeratin-positive cells that stain positive for the proliferation marker.

TABLE 2

SIMULTANEOUS QUANTITATIVE ANALYSIS OF DNA AND G-ACTIN AT THE SINGLE CELL LEVEL IN BREAST CANCER CELL LINES

DNA	T47D	MCF-7	Control**
Cell DNA / Diploid Cell DNA Median	1.7	2.2	1.1 1.2
Percentage of cells with a ratio of two or greater	16/54=30%	30/40=75%	4/52=7.7% 0/43
Percentage of cells with a ratio of 1.4 or greater	51/54=94%	100%	16/52=31%

ACTIN	T47D	MCF-7	Control**
Cell Actin / WBC Actin Median	3.6	2.25	Not Done
Percentage of cells with a action ratio above median and a DNA ratio of two or greater	15/16=94%	23/30=77%	----

** Control is normal epithelial cells of prostate origin

Table 3: Recovery

Breast Cancer T47D Cells — Viability 89%

Twin Spikes	I	II	III	IV	V	VI	VII	Negative Control	Mean	SD	%CV
1-Spot Count No Isolation	100	106	94	110	96	107	114	none	103.8	7.4	7.2%
2-Recovered from Isolation	79	70	64	91	67	64	83	none	74.0	10.5	14.2%
CK-KS staining	+++	+++	+++	+++	+++	+++	+++				
DAPI staining	++	++	++	++	++	++	++				
% Recovery	79%	66%	68%	82%	70%	60%	73%		71.1%	7.6	10.7%

Breast Cancer MCF-7 Cells — Viability 91%

Twin Spikes	I	II	III	IV	V	VI	VII	Negative Control	Mean	SD	%CV
1-Spot Count No Isolation	63	69	59	72	64	66	68	none	65.9	4.3	6.5%
2-Recovered from Isolation	50	44	30	40	46	55	51	none	45.1	8.3	18.4%
CK-KS staining	+++	+++	+++	+++	+++	+++	+++				
DAPI staining	++	++	++	++	++	++	++				
% Recovery	79%	64%	51%	56%	72%	83%	75%		68.6%	11.8	17.3%

ExtractData of PDX060601A prepared by ZPLum

7. KEY RESEARCH ACCOMPLISHMENTS

- Developed and tested a new antibody cocktail for detection of circulating epithelial cells.

- Validated test for circulating epithelial cells at our new location with respect to recovery of breast cancer cells from spiked blood samples using the reformulated antibody cocktail.

- Demonstrated feasibility for the simultaneous measurement g-actin and DNA content at the single cell level by quantitative fluorescence image analysis as a breast cancer marker.

- Initiated a study to determine if Fas-mediated apoptosis is abrogated in breast cancer cell lines.

- Showed that two different markers for cell proliferation are able to label the same breast cancer cells.

8.   REPORTABLE OUTCOMES

- U.S. Patent Application Serial No. 09/430,175, filing date of October 29, 1999, entitled "Multiple Marker Characterization of Single Cells."

9. CONCLUSIONS

- Reformulated antibody cocktail for staining epithelial cells gives a much brighter fluorescence signal and has the potential for identifying cells not previously detected.

- Test for circulating breast cancer cells has been re-established and validated at our new location.

- Quantitative fluorescence image analysis of g-actin and DNA content is a potential marker for individual breast cancer risk assessment.

- Either an indicator of DNA synthesis (thymidylate synthase) or the nuclear antigen (Ki67) can be used as a marker for breast cancer cell proliferation.

10. REFERENCES (Copies are supplied after Exhibits in Appendices Section 11).

- Ts'o, POP, et al., "Blood Biopsy for Epithelial Cancer Cells Based on Circulating Cancer Cell Tests," 92nd Annual Meeting Proceedings of the American Association for Cancer Research, Vol.. 42, March 27, 2001.
- Ts'o, POP, et al., "Blood Biopsy for Epithelial Cancer Cells Based on Circulating Cancer Cell Tests," Abstract, 92nd Annual Meeting in Proceedings Supplement, pp. 78-79, numbered LB35, March 2001.
- "Taking the Stealth out of Cancer's Spread," BusinessWeek Online News Flash, March 28, 2001.
- "Blood Test Detects Epithelial Cancer Cells," Oncology News International and Primary Care & Cancer, June 2001.
- Lesko, S, et al., "A New Analytical Method To Measure DNA Aneuploidy in Circulating Cancer Cells," Abstract to be presented at the American Association of Clinical Chemstry, Chicago, Illinois, August 2001.
- Lauderdale, VR, et al., "Detection and Imaging of Prostate, Breast, Colon, Gastric, and Liver Cancer Isolated form Blood through Use of Fluorescent-Labeled Epithelium-Specific and Epithelium-Carcinoma Antibody Stains," Abstract to be presented at the American Association of Clinical Chemstry, Chicago, Illinois, August 2001.

11. APPENDICES

See attached pages 16 to 24 (Blood Biopsy Booklet)

Figure Legends

Figure 1   Color composite image of a MCF-f breast cancer cell showing positive staining for both thymidylate synthase and the nuclear antigen, Ki67. Anti-ki67-CY3 is shown in red; anti-thymidylate synthase-Cy5 is shown in green.

Figure 2   Monochrome images of the same field of T47D cells stained simultaneously with thymidylate synthase-CY5 and ki67-CY3. Left-CY5 image; right-CY3 image.

Figure 3   Color composite image of T47D cells stained simultaneously with anti-cytokeratin-FITC and DNAase-CY5. DAPI is present in the mounting medium. Anti-cytokeratin is shown in green; g-actin-bound DNAase is shown in red; DNA is shown in blue. Pink nuclei result when pseudocoloring is used to depict blue nuclear DNA and the red g-actin in the same nucleus.

Figure 4   Color composite image showing apoptotic cells induced by 1uM staurosporine. Apoptotic cells have fragmented chromatin and have incorporated dUTP-FITC at DNA strand breaks. DAPI –stained nuclei-blue, positive Tunel assay-green.

Figure 5   Monochrome images of the same field of T47D cells showing apoptotsis induced by 1 ug / ml of antihuman Fas IgM. Left- image showing chromatin fragmentation in DAPI-stained nuclei; Right- FITC image showing incorporation of dUTP-FITC into DNA strand breaks by terminal nucleotidyl transferase (Tunel assay).

FIGURE 1
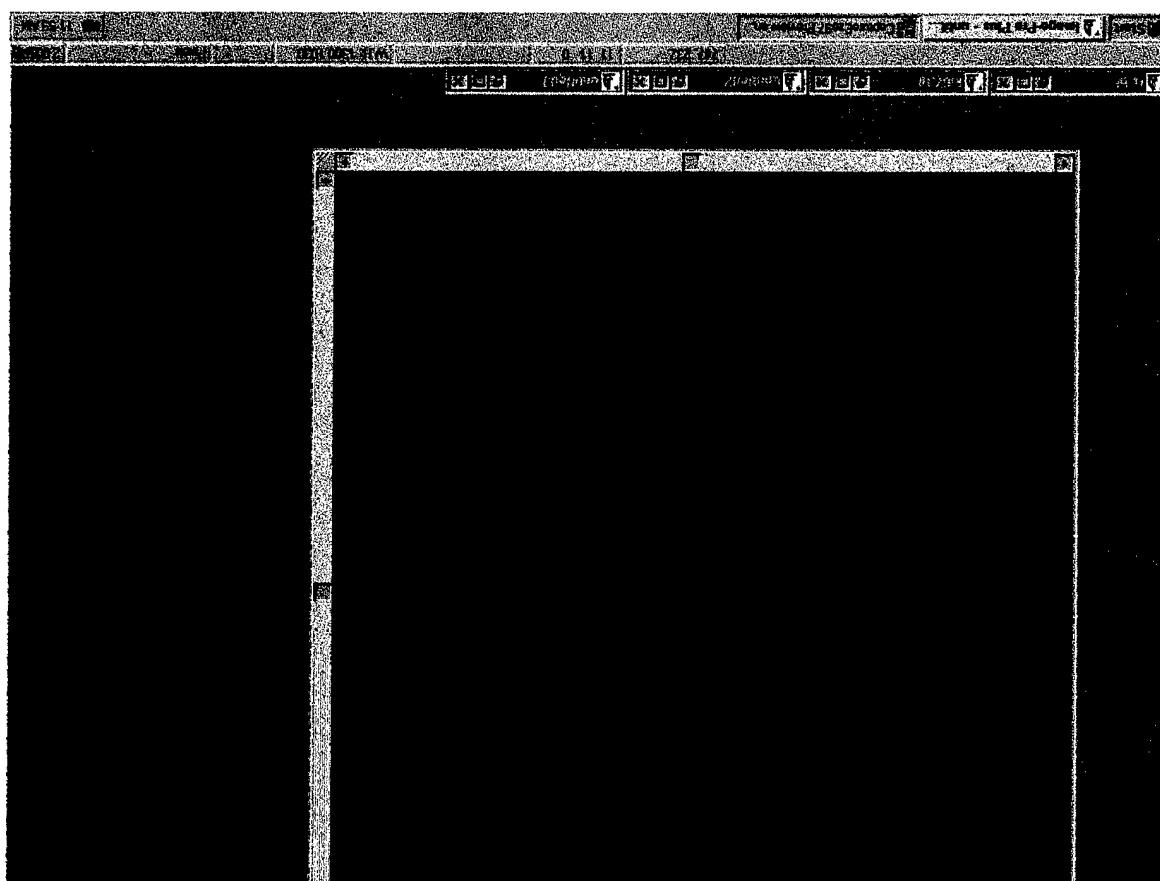
FIGURE 2
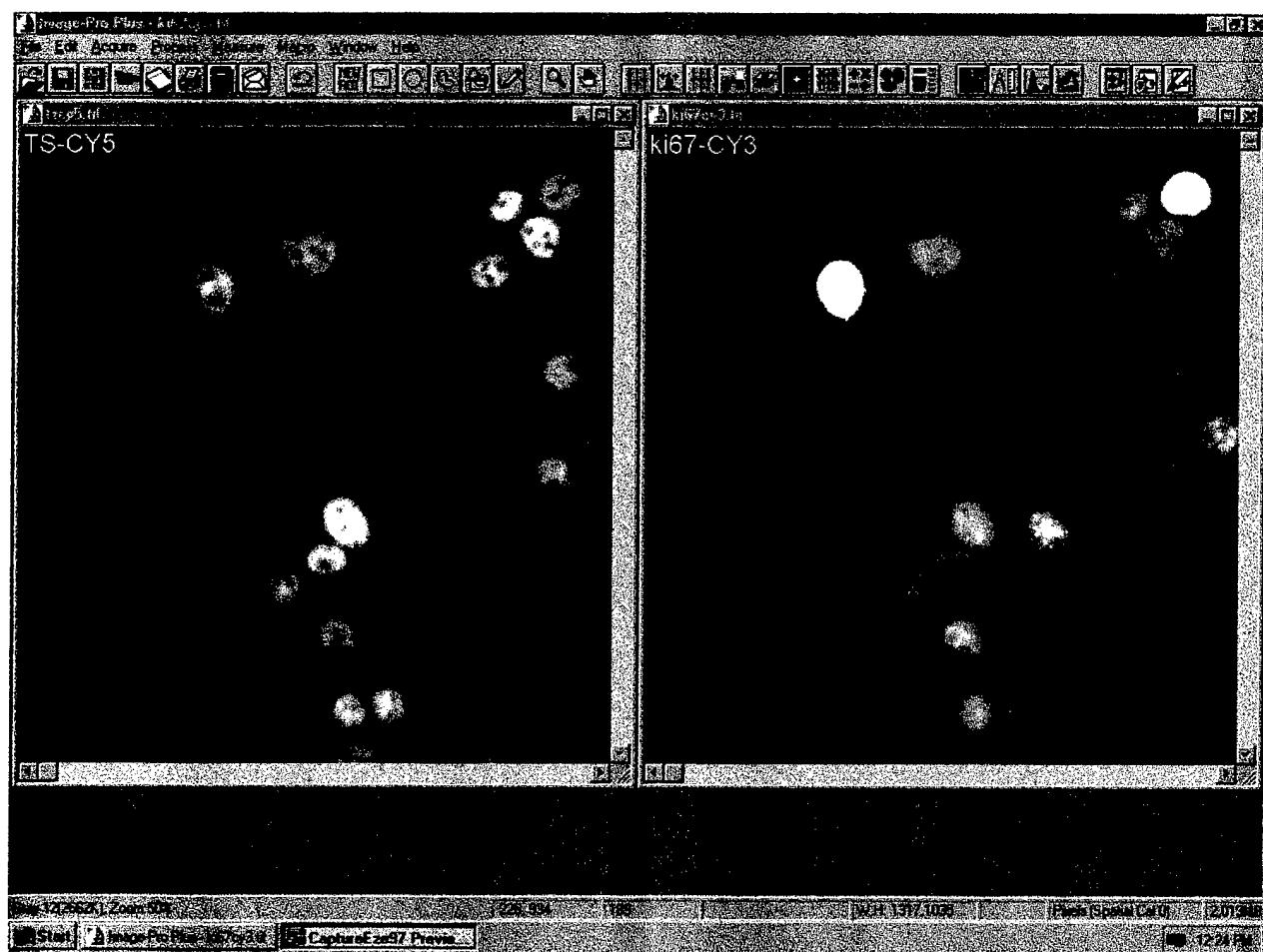
FIGURE 3


T47D
green-CK
actin-red
DNA -blue

FIGURE 4

apoptotic mcf-7
green-positive Tunel
DNA -blue
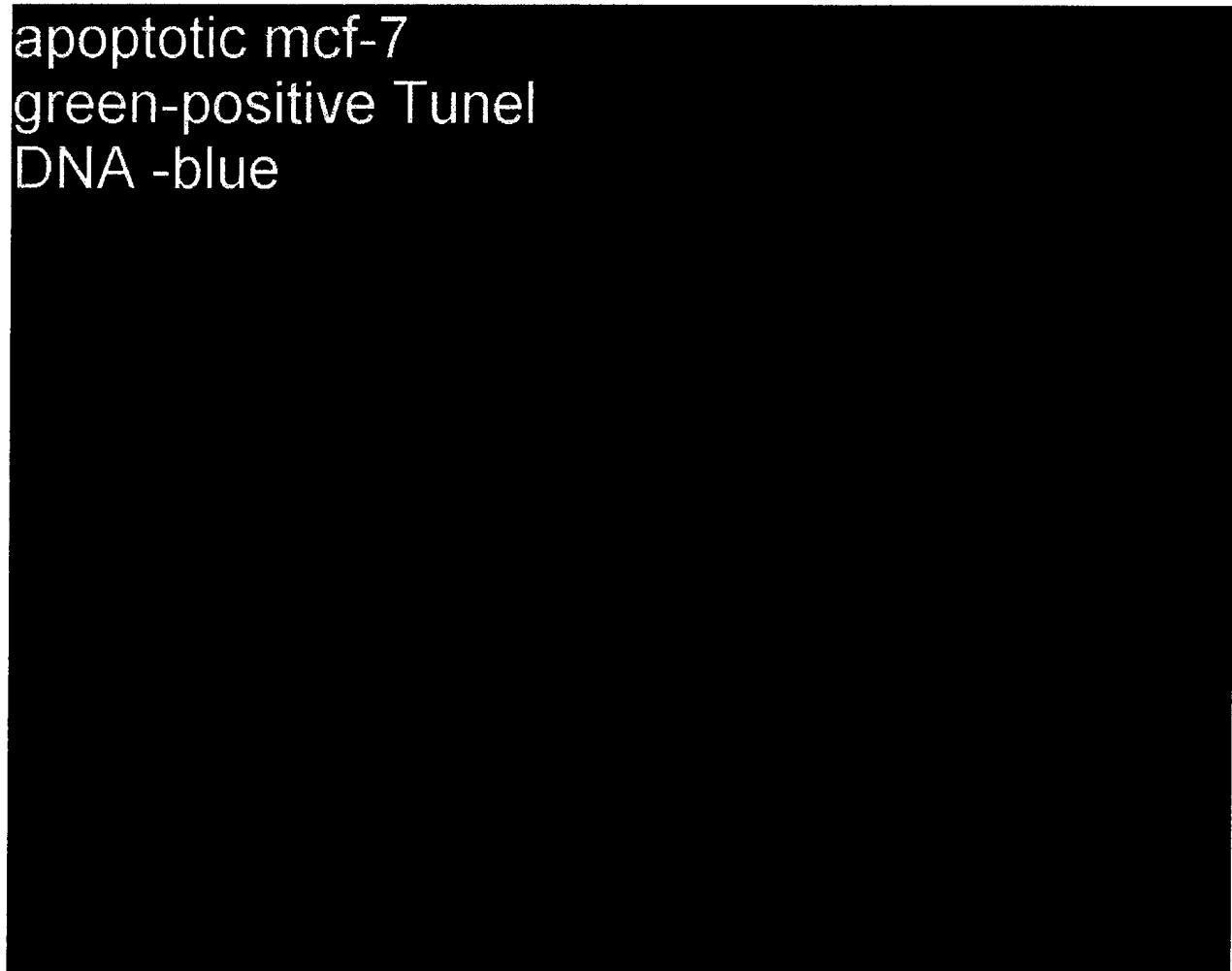
FIGURE 5
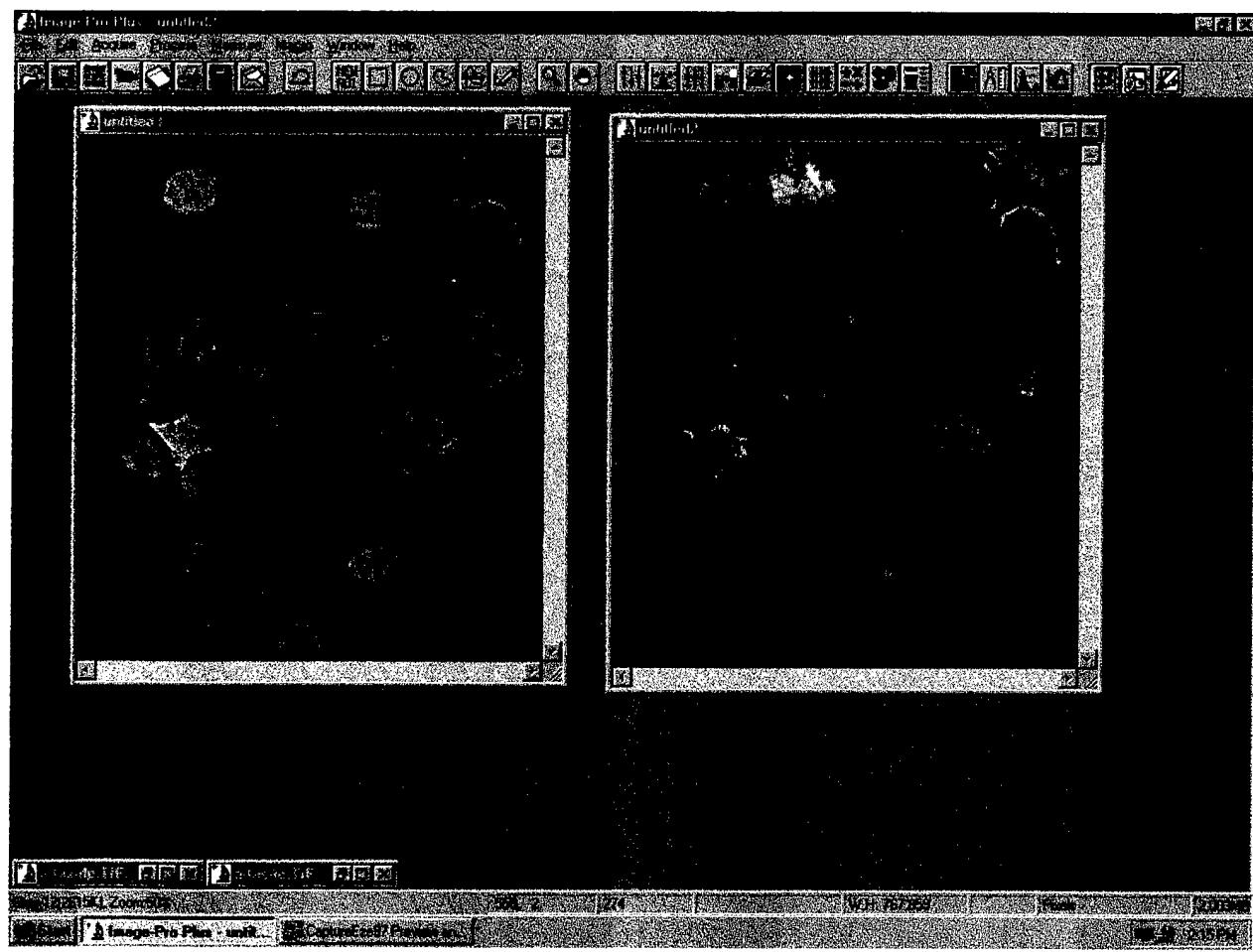
Detection and Imaging of Prostate, Breast, Colon, Gastric, and Liver Cancer Isolated from Blood through Use of Fluorescent-Labeled Epithelium -Specific and Epithelial-Carcinoma Antibody Stains

V. R. Lauderdale[1], C. Chisholm[2], Z. P. Lum[2], W. Ching[2], Y. Zhou[2], D. Ertel[2], E. Principe[2], S. Clinton[2], P. Ts'o[2], [1]Cell Works, Inc.: Baltimore, MD ; [2]Cell Works Inc.: Baltimore, MD

Presentation Number: 445

Keywords: cancer detection, circulating cancer cells, epithelial cell detection

Objective: The objective of this study was to qualify a mix of fluorescent-tagged epithelium and cancer specific antibodies to detect, quantify and image intact prostate, breast, colon, gastric, and liver cancer cells isolated from blood. Relevance: 80% of all cancers are epithelial in origin. A test developed to detect epithelial cells and/or carcinoma glycoprotein on cells found in blood provides another diagnostic tool to screen for cancer spreading through the circulatory system, thought to be a primary route of metastasis. Methods: CK-KS Stain was designed as a reagent with primary reactivity to keratin proteins #4,5,6,8,10,13,18,19 (CK) and tumor-associated glycoprotein (KS) expressed on human carcinomas. A test was designed to identify intact cancer cells from blood with three primary steps. 1) Concentrate cancer cells from a 15-20 ml blood sample, using double-gradient centrifugation and CD 45 beads to eliminate blood cells. 2) Stain with fluorescent labeled CK-KS Stain 3) Fluorescent microscope search, detection and imaging. In order to verify that the stain would react with a number of cancer cell lines, cultured cells from four types of cancer were quantitatively placed on slides, stained with the CK-KS Stain, imaged and counted. Additionally, to validate that cancer cells in blood could be quantitatively recovered using the enrichment steps and CK-KS Stain together, several types of cultured cancer cells were quantitatively spiked into blood samples, with subsequent enrichment, staining and microscope detection. Prostate cell lines (TSU, PC-3, DU 145, LNCAP), breast cancer cell line (MCF-7), gastric cancer cell line (KATO III) and liver cancer cell line (HEP3B) cancer cells were mixed with human blood and added to slides in a quantitative manner ranging 7 to 45 cancer cells, then fixed and stained with the CK-KS stain. The nuclear morphology of each cancer cell line was identified and counted as the gold standard. The readability of the CK-KS stain in the cancer cell cytoplasm and cell surface was the test parameter. Results: All cell lines showed good quality staining. With the exception of one cell line (Hep3b) which had a recovery of 97%, all other cancer cell lines had recoveries of 100%. White blood cells, as negative controls were 100% negative. For validating the total test (isolation, staining and microscopy), two lines of cultured cancer cells each, from prostate, breast and colon cancer, were spiked into blood at 20 to 40 cells per 15-20 ml sample. Two to three recovery runs were done per cell line (N=5). Within-run average recovery ranged from 63% to 78% (SD = 5% to 15%) and within-run CV% from 8% to 19%. Negative controls (blood without spiked cancer cells) were 100% negative. Additionally, cancer cells were isolated from the blood of prostate and breast cancer patients, with results ranging from 0 to 200 cancer cells detected. Conclusion: This study demonstrates the feasibility of detecting six types of epithelial origin cancer cells in patient's blood, using the CK-KS fluorescent stain and the double gradient isolation system, with average recoveries ranging from 63% to 78%.

A New Analytical Method To Measure DNA Aneuploidy In Circulating Cancer Cells

S. Lesko[1], K. Ohara[2], P. Ts'o[2], V. Lauderdale[2], X. Jin[2], D. Ertel[2], *[1]Cell Works, Inc.: Baltimore, MD ; [2]Cell Works Inc.: Baltimore, MD*

Presentation Number: 464

Keywords: Cancer, Aneuploidy, Circulating Cancer Cells

Objective: Aneuploidy is an excellent marker for identifying cancer cells (CC). The objective of this study was to test the feasibility of measuring the ratio of DNA content in circulating epithelial cancer cells (CEC) isolated from prostate cancer patient's blood to DNA of white blood cells (WBC's), as an indicator of cancer cell aneuploidy. Relevance: Most circulating WBC's are in the G0 phase of the cell cycle and have one pair of each chromosome, i.e., a DNA content of 2N. Dividing CEC's, (G2-M phase), can have DNA content of up to 4N. Therefore, a CEC/WBC DNA content ratio greater than 2.0 is a measure of aneuploidy. The basis for this quantitative DNA assay is a comparison of the DNA content of reference WBC's, with that of circulating epithelial cells (CEC) isolated from patient's blood. Methods: Fluorochrome, 4-6-diamidino-2-phenylindole (DAPI), binds to DNA with high specificity. The complex exhibits intense fluorescence, which can be quantified as integrated fluorescence intensity (IFI), using a sophisticated computerized fluorescence microscope. To validate the assay, cultured cancer cells (CC) were mixed with blood, fixed, and stained with DAPI. As a negative control for aneuploidy, normal prostate cancer cells were held 24 hours without growth factors to inhibit cell division. CC/WBC IFI ratio was measured. Additionally, CEC's were isolated from two late stage prostate cancer patients, and the CEC/WBC IFI ratio measured. Results: All cell lines had CC/WBC IFI ratio ranges greater than 2.0, half had a median ratio greater than 2.0 and the remainder had median ratios between 1.4 and 1.7. Normal prostate cells (G0/G1 phase) had a median ratio of 0.98. Median CEC/WBC IFI ratios, from two advanced prostate cancer patients, were 1.6 and 1.4.(ranges listed in table). Conclusion: CEC whose CEC:IFI ratio is greater than two should be considered neoplastic.

Cancer/WBC Integrated Fluorescent Intensity Ratios

Test: Cancer/Patient/Control	Number Cells Measured	Median CC:WBC IFI Ratio	Range of Ratios
Breast Cancer Cell Line MCF-7	55	2.02	1.5-3.5
Breast Cancer Cell Line T47D	45	1.65	1.2-3.5
Prostate Cancer Cell Line LnCAP	16	2.50	1.9-4.4
Prostate Cancer Cell Line TSU	13	2.05	1.6-3.4
Colon Cancer Cell Line HT-29	31	1.71	1.4-3.7
Colon Cancer Cell Line HCT-116	22	2.20	1.4-4.0
Gastric Cancer Cell Line Kato III	25	2.50	1.8-4.9
Gastric Cancer Cell Line AGS	21	1.58	1.2-2.9
Liver Cancer Cell Line HEP-G2	57	1.40	1.1-2.7
Liver Cancer Cell Line SK-HEP	82	1.60	1.2-3.5
Prostate CEC-Patient A	9	1.40	1.2-2.8
Prostate CEC-Patient B	14	1.60	1.3-2.4
Normal Prostate Cell Line (Non-cancer & Not dividing)	30	0.98	0.9-1.2

"BLOOD BIOPSY" FOR EPITHELIAL CANCER CELLS BASED ON CIRCULATING CANCER CELL TESTS

Ts'o, Paul O.P.; Lesko,Steve; Lauderdale,Vivian; Ohara, Karen
Cells Works Inc., Baltimore, MD 21224

This publication was submitted to the American Association for Cancer Research (AACR) by Dr. Paul Ts'o et.al., of Cell Works Inc. as a late breaking research abstract and was selected for presentation by the AACR during its 92nd Annual Meeting. AACR is one of the most important and prestigious cancer research organizations in the United States and the world. In addition to the honor of presenting the publication at the meeting, Cell Works submission was further selected by AACR for their press conference on March 27, 2001. Only three presentations were selected for this conference, and the publication of Cell Works was selected as the outstanding representative for Clinical/Medical Studies. The abstract of the presentation, as well as the photo of Dr Ts'o in the press conference, can be found in the web site of AACR at http://aacr01.agora.com/planner/displayabstract.asp?presentationid=19423.

The abstract is cited in the AACR 92nd Annual Meeting in the Proceedings Supplement, pp. 78-79, abstract number LB35.

This publication represents the entire contents of the abstract and the poster presentation.

Paul Ts'o, Ph.D. answers questions from the press at the Late-Breaking Research press conference

Publications

Ts'o, P., Lesko, S., Lauderdale, V., Ohara K., "Blood Biopsy for Epithelial Cancer Cells Based on Circulating Cancer Cell Tests"AACR 92nd Annual Meeting in Proceedings Supplement, Vol.42, pp. 78-79, abstract LB35,(2001)

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"Blood Biopsy" for epithelial cancer cells based on circulating cancer cell tests.

Ts'o, Paul O.P.; Lesko, Steve; Lauderdale,Vivian; Ohara, Karen; Cell Works, Inc., Baltimore, MD 21224

The importance of early detection of cancer recurrence after treatment of a primary tumor, early diagnosis of metastasis, and early information about the responsiveness to systemic chemical/hormonal therapies, are well recognized for management of cancer patients. By spiking cultured cancer cells from more than thirty cancer cell lines of Prostate, Breast, Colon, Gastric, Liver, Kidney cancers, etc. into 20 ml of human blood, Cell Works has established a universal procedure, the Circulating Cancer Cell (CCC) Test to detect circulating epithelial cancers. This procedure utilizes a double gradient sedimentation for the removal of most RBC and WBC as well as magnetic cell sorting for the additional removal of WBC before spreading the cancer cells onto a slide utilizing a cytospin apparatus. The fixed cells on the slide are then stained with various specific molecular probes, with selected fluorescent dyes attached. These cells are automatically scanned with an award winning spectroscopic microscope system, first in low magnification, where the fluorescent digital image is captured at a resolution of 0.2 um using multiple excitation/emission wavelengths, then at higher resolution for further analysis. The system has automatic adjustment of exposure, focus and other parameters required for proper image acquisition and analysis to identify cancer cells and markers on the basis of intensity and blob analysis. Image storage and records are automatically kept, maintained on a case by case basis for production of print outs and reports. This test can routinely provide five types of basic information about individual cancer cells found in the blood sample:

1. The number of epithelial cells by the use of a cocktail of 4 types of monoclonal antibody probes for cytokeratins and tumor specific surface antigens. The recovery rate is usually 50-80%. Sensitivity experiments, where 10 cancer cells are added to 20 ml of blood (which contains 120-200 million WBC), show that at least 5 cancer cells would be detected. Preliminary data on blood samples from 13 prostate cancer patients indicate that if there are 3000 circulating cancer cells in the whole body, several cancer cells would be detected in the 20 ml of sample.
2. These epithelial cells in the blood have abnormally high DNA content when compared to the normally diploid WBC of the same patient on the same slide. The ratio of CCC/WBC DNA content is in the range of 1.5-3.0, indicative of aneuploidy and polyploidy, which are indications of neoplasia. These epithelial cells have an abnormally large two dimensional nuclear area (flattened volume).
3. These epithelial cells are analyzed for thymidylate synthetase or DNA fragmentation as an indication of active growth (correlated with high levels of thimidylate synthetase content) or cell death (by measuring DNA fragmentation via tunel assay). These two measurements are being simultaneously performed on the same slide in order to identify and compare the growing cells versus the dying ones.
4. Information is generated about gene copy numbers or specific chromosome numbers of these epithelial cells , such as androgen receptor gene copy number or number of chromosomes 8,7,18, etc. for genomic/chromosomal abnormalities
5. Absence or the presence of certain proteins such as androgen receptors, estrogen receptors, progesterone receptors, Vitamin D receptors, etc. can be analyzed, which provides valuable information for patient therapy design and management. Thus, information about the circulating cancer cells for 6-8 epithelial cancer obtained from this "Blood Biopsy" will provide valuable, previously unavailable data to the attending physicians of these patients.

Thus, information about the circulating cancer cells for 6-8 epithelial cancer obtained from "Blood Biopsy" will provide valuable, heretofore, unavailable data to the attending physicians of these patients. Active clinical studies in providing valuable data on how to utilize this technology of CCCT are in progress.

In addition, the CCCT will provide very valuable information for basic oncology research about leakage of cancer cells from the primary tumors and the secondary tumors into circulation as well as the fate of these circulating cancer cells in the body. In a recent publication (Cancer, 88: 2787-2795, 2000), we provide evidence which strongly suggests that the circulating cancer cells can divide and multiply in the blood, and this information was not known before. These circulating microtumors can be the initial bridgehead for metastasis.

The importance of early detection of cancer recurrence after treatment of a primary tumor, early diagnosis of metastasis, and early information about the responsiveness to systemic chemical/hormonal therapies, are well recognized for management of cancer patients. By spiking cultured cancer cells from more than thirty cancer cell lines of Prostate (see Table 1), Breast (see table 2), Colon (see Table 3), Gastric, Liver, Kidney cancers, etc. into 20 ml of human blood, Cell Works has established a universal procedure, the Circulating Cancer Cell (CCC) Test to detect circulating epithelial cancers.

Table 1: Prostate Cancer Validation GP017 Start Date: 08-30-99

	Run 1			Run 2		
Sample #	Cell Type	# cells/sample	% recovery	Cell Type	# cells/sample	% recovery
1	LNCAP	23	65	TSU	27	71
2	LNCAP	23	65	TSU	27	64
3	LNCAP	23	96	TSU	27	78
4	LNCAP	27	68	TSU	49	63
5	LNCAP	27	74	TSU	49	65
6	LNCAP	27	76	TSU	49	73

Within-Run Average Recovery % 74.0	Within-Run Average Recovery % 69.0
Within-Run standard Deviation 11.7	Within-Run standard Deviation 6.0
Within-Run Coefficient of Variation 15.8	Within-Run Coefficient of Variation 8.6

Run to Run Average Recovery %   71.5
Run to Run Standard Deviation %   9.2
Run to Run Coefficient of Variation 12.9

Table 2: Breast Cancer Validation GP022 Start Date: 05-26-00

Sample #	Run 1				Run 2			Run 3		
	Cell Type	# cells/ sample	% recovery	Cell Type	# cells/ sample	% recovery	Cell Type	# cells/ sample	% recovery	
1	T47D	20	60	MCF7	20	87	T47D	22	55	
2	T47D	20	100	MCF7	13	57	T47D	22	59	
3	T47D	20	85	MCF7	14	61	T47D	22	68	
4	T47D	40	68	MCF7	35	76	MCF7	22	59	
5	T47D	40	68	MCF7	26	57	MCF7	22	82	
6	T47D	40	68	MCF7	39	85	MCF7	22	59	
Within-Run Average Recovery %		74.8			70.5			63.7		
Within-Run standard Deviation		14.8			13.9			10.0		
Within-Run Coefficient of Variation		19.8			19.7			15.6		

Run to Run Average Recovery %   69.7
Run to Run Standard Deviation %   13.2
Run to Run Coefficient of Variation   18.9

Table 3: Colon Cancer Validation GP025 Start Date: 08-18-00

Sample #	Run 1				Run 2			Run 3		
	Cell Type	# cells/ sample	% recovery	Cell Type	# cells/ sample	% recovery	Cell Type	# cells/ sample	% recovery	
1	HT-29	25	72	T84	24	75	HT-29	22	59	
2	HT-29	25	56	T84	24	79	HT-29	22	73	
3	HT-29	25	64	T84	24	63	HT-29	22	64	
4	HT-29	50	68	T84	48	77	T84	21	57	
5	HT-29	50	82	T84	48	79	T84	21	81	
6	HT-29	50	88	T84	48	69	T84	21	71	
Within-Run Average Recovery %		71.7			73.7			67.5		
Within-Run standard Deviation		11.8			6.4			9.2		
Within-Run Coefficient of Variation		16.4			8.7			13.6		

Run to Run Average Recovery %   70.9
Run to Run Standard Deviation %   9.2
Run to Run Coefficient of Variation   13.0

This procedure utilizes a double gradient sedimentation for the removal of most RBC and WBC as well as magnetic cell sorting for the additional removal of WBC before spreading the cancer cells onto a slide utilizing a cytospin apparatus. The fixed cells on the slide are then stained with various specific molecular probes, with selected fluorescent dyes attached. These cells are automatically scanned with an award winning spectroscopic microscope system (see below), first in low magnification, where the fluorescent digital image is captured at a resolution of 0.2 um using multiple excitation/emission wavelengths, then at higher resolution for further analysis. The system has automatic adjustment of exposure, focus and other parameters required for proper image acquisition and analysis to identify cancer cells and markers on the basis of intensity and blob analysis. Image storage and records are automatically kept, maintained on a case by case basis for production of print outs and reports (see FIG.1).

UMBC News Release
May 3, 1999

CELL WORKS WINS SMITHSONIAN HONOR

Baltimore Biomedical Company's Automated Microscope Added to National Museum of American History's Permanent Collection

"Baltimore – An automated microscope developed by Cell Works, Inc., a biomedical company located at the University of Maryland, Baltimore County (UMBC), has won a place in the Permanent Research Collection on Information Technology at the Smithsonian Institution" National Museum of American History..."

Fig. 1

CCCT Operations
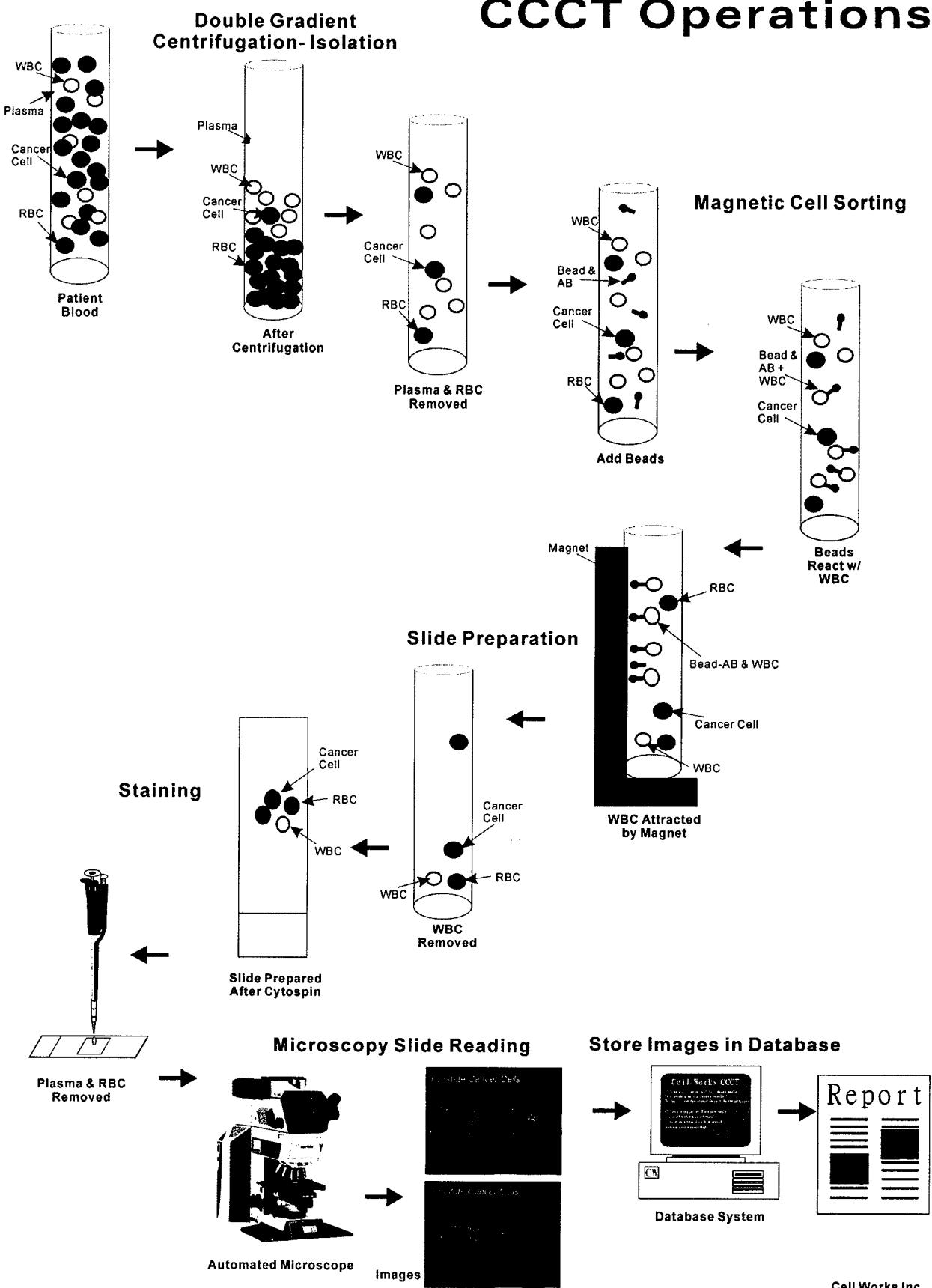
Double Gradient Centrifugation- Isolation

Magnetic Cell Sorting

Slide Preparation

Staining

Microscopy Slide Reading

Store Images in Database

Report

This test can routinely provide five types of basic information about individual cancer cells found in the blood sample:

1.   The number of epithelial cells by the use of a cocktail of 4 types of monoclonal antibody probes for cytokeratins and tumor specific surface antigens. The recovery rate is usually 50-80%. Sensitivity experiments, where 10 cancer cells are added to 20 ml of blood (which contains 120-200 million WBC), show that at least 5 cancer cells would be detected. Preliminary data on blood samples from 13 prostate cancer patients indicate that if there are 3000 circulating cancer cells in the whole body, several cancer cells would be detected in the 20 ml of sample.

DETECTION OF EPITHELIAL CELLS

A cocktail of carefully selected monoclonal antibodies was developed for staining and detecting most types of epithelial cancer cells and to provide a good signal-to-noise ratio. The cocktail recognizes at least nine different cytokeratin peptides and a tumor-associated glycoprotein, all of which are labeled with FITC. This cocktail has been used to identify at least two lines of cultured cancer cells from prostate, breast and colon tumors. Average recovery ranged from 63% to 78%. A typical image is shown below (see FIG.2).
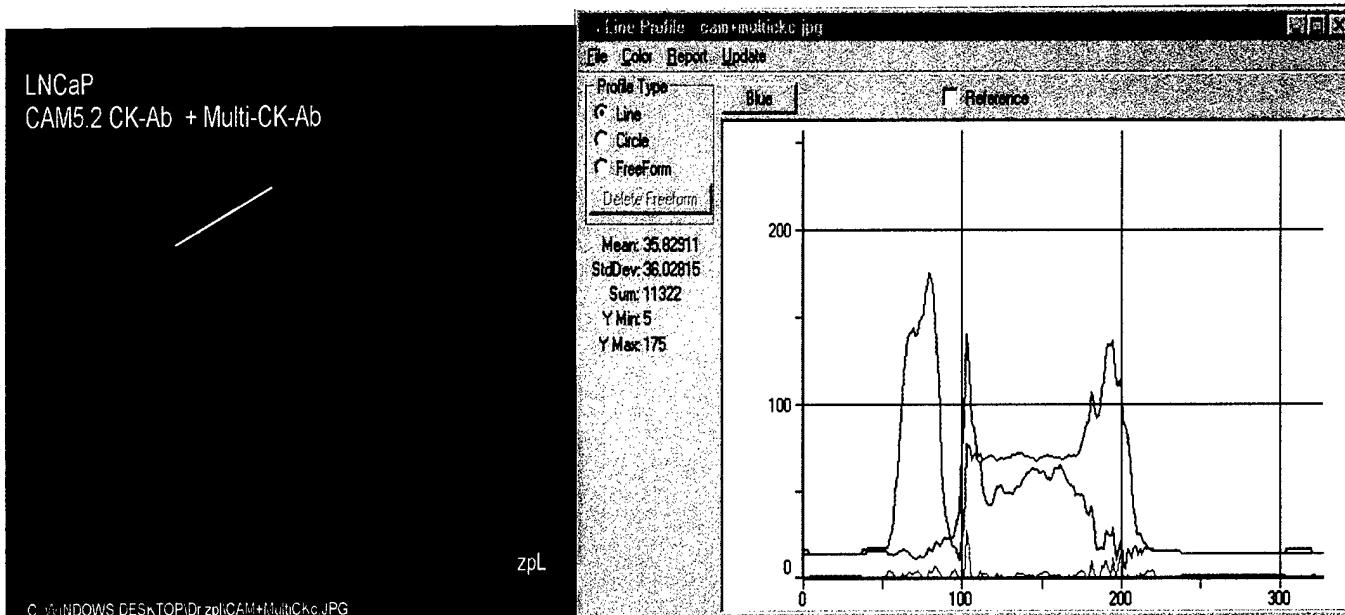
FIG.2 The microscope can perform quantitative spectral-spatial analyses of the image. Y-axis: Fluorescent signal intensity. X-axis: spatial parameter, cell size.

DETECTION LIMIT OF CCCT

Sensitivity of the CCCT has been determined by obtaining both a 20 ml peripheral blood sample and a leukapheresis sample (equivalent to 3 liters of blood) from each of 13 different patients. The data show that several cancer cells can be detected in a 20ml blood sample if there are approximately 3000 cancer cells circulating in the body, i.e., in 6 liters of blood.

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2. These epithelial cells in the blood have abnormally high DNA content when compared to the normally diploid WBC of the same patient on the same slide (see FIG.3). The ratio of CCC/WBC DNA content is in the range of 1.5-3.0, indicative of aneuploidy and polyploidy, which are indications of neoplasia (see Table 4). These epithelial cells have an abnormally large two dimensional nuclear area (flattened volume).
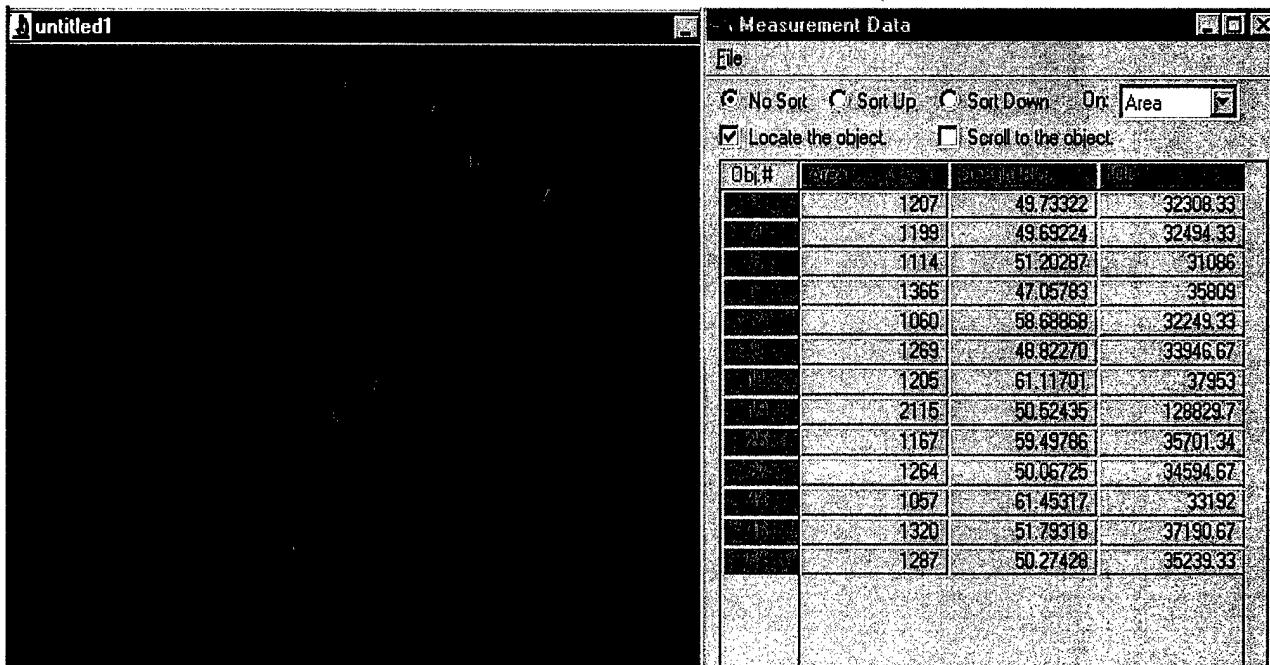

FIG.3 The microscope can perform quantitative spectral-spatial analyses of signals from a defined area.

NUCLEAR DNA RATIO

Cancer Cell (#19) Integrated DNA Fluorescence Intensity is **128830** = **3.75**
Mean of 12 WBC's Integrated DNA Fluorescence Intensity is **34313**

Table 4: DNA CONTENT IN CANCER CELL LINES / REFERENCE WBCs

		Number of Cells Analyzed	Median Ratio	Range of Ratios
Breast	MCF-7	55	2.02	1.5-3.5
	T47D	45	1.65	1.2-3.5
Prostate	LnCap	16	2.5	1.9-4.4
	TSU	13	2.05	1.6-3.4
Colon	HT-29	31	1.71	1.4-3.7
	HCT-116	22	2.2	1.4-4.0
Gastric	Kato III	25	2.5	1.8-4.9
	AGS	21	1.58	1.2-2.9
Liver	HEP-G2	57	1.4	1.1-2.7
	SK-HEP	82	1.6	1.2-3.5
Normal Prostate*		30	0.98	0.9-1.2

*Held 24 hours in the absence of growth factors

3. These epithelial cells are analyzed for thymidylate synthetase or DNA fragmentation as an indication of active growth (correlated with high levels of thimidylate synthetase content) or cell death (by measuring DNA fragmentation via tunel assay). These two measurements are being simultaneously performed on the same slide in order to identify and compare the growing cells versus the dying ones (see FIG.4, FIG.5 and FIG.6).


TS + TUNEL
TS-CY5 (Red)
TUNEL-FITC (Green)
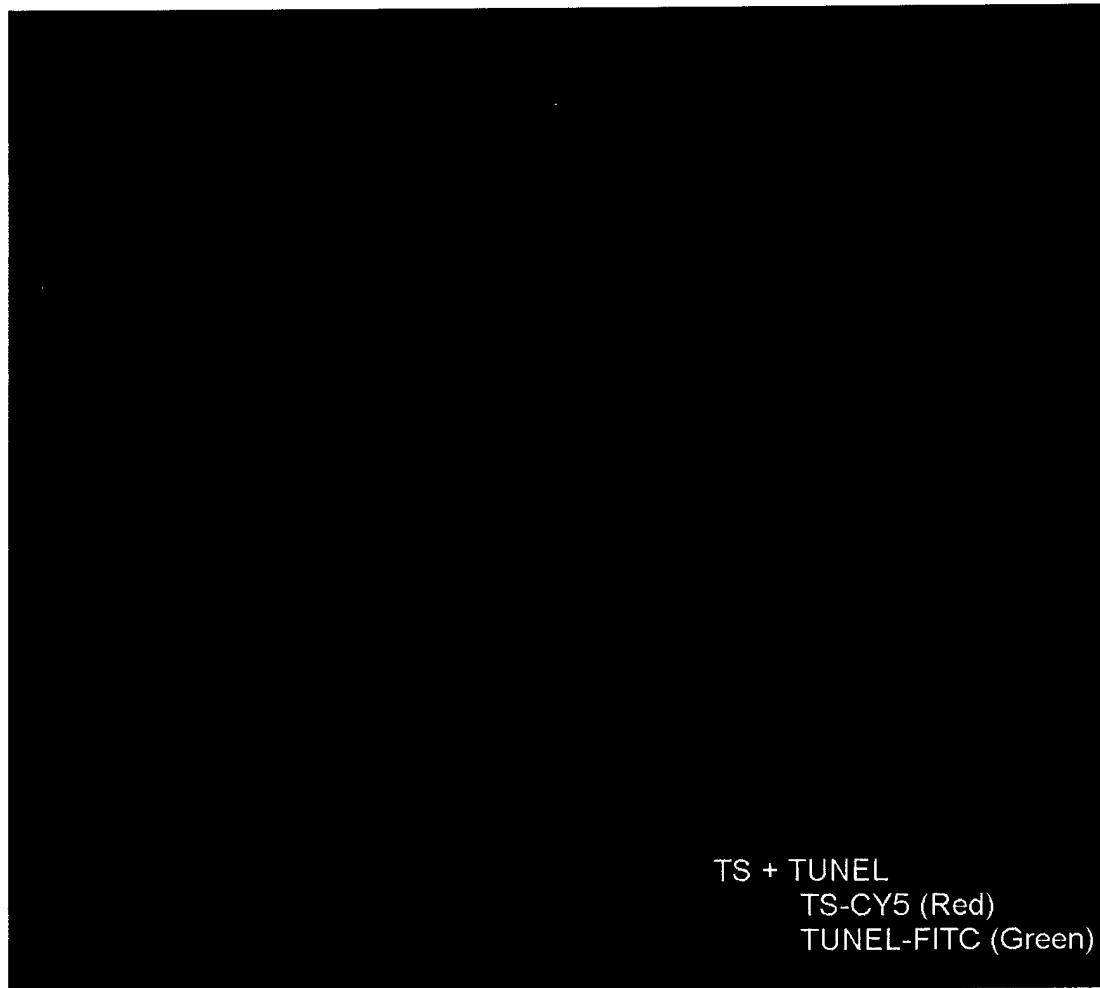
FIG.4 Thymidylate synthetase (red), TUNEL (green) positive control, showing strand breaks induced by DNase treatment.
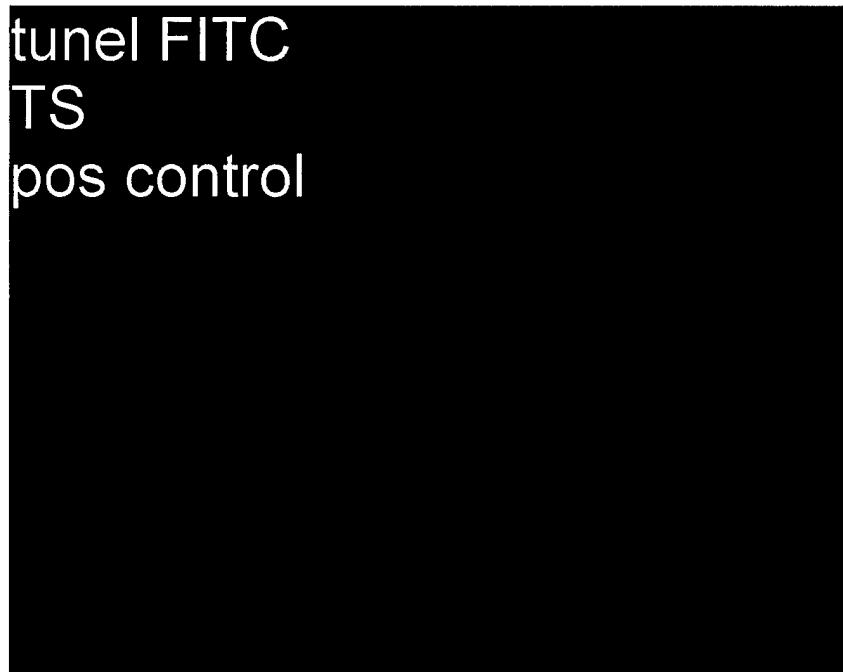
FIG.5 LnCAP cells treated as a positive control for DNA fragmentation. Thymidylate synthetase (TS) (red), TUNEL (green) positive control, showing strand breaks induced by DNase treatment.
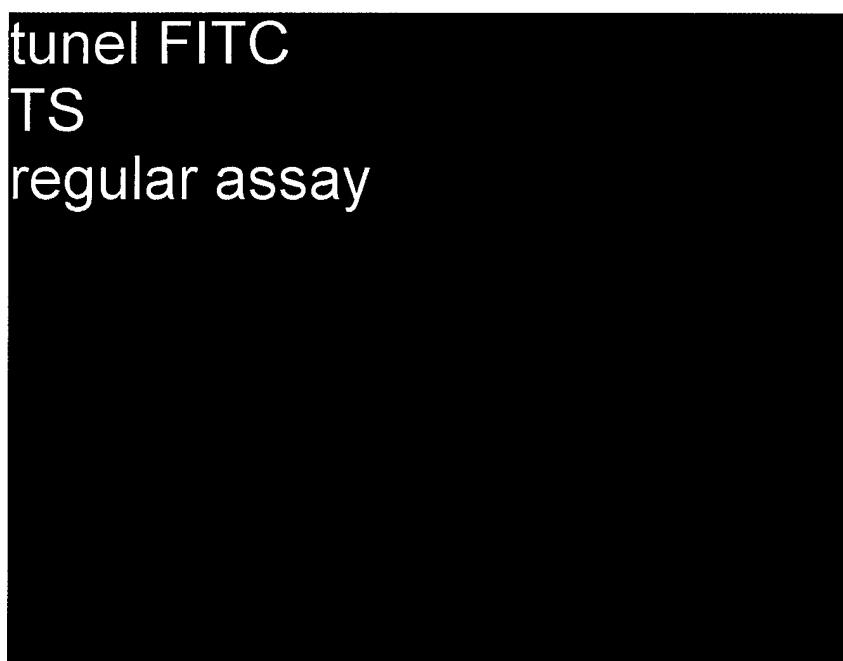
FIG.6 LnCAP cells, thymidylate synthetase (TS) (red), TUNEL (green).

4.   Information is generated about gene copy numbers or specific chromosome numbers of these epithelial cells , such as androgen receptor gene copy number or number of chromosomes 8,7,18, etc. for genomic/chromosomal abnormalities (see FIG.7 and FIG.8).
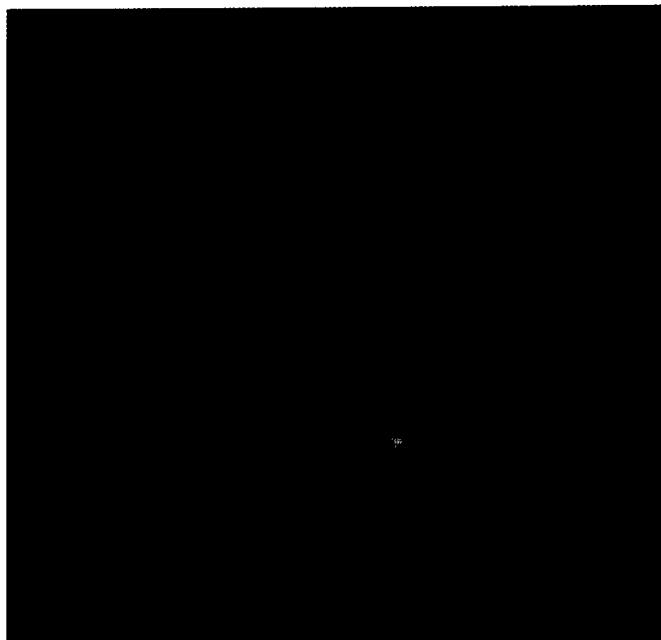
FIG.7   Prostatic cells from the blood of prostatic cancer patient were stained by in situ hybridization with chromosome 7 (blue) and 8 (red) centromere probes in nucleus and by immunocytochemistry with PSA antibody (green) in cytoplasm. X 6000

FIG.8   White blood cell from blood of the prostatic cancer patient were stained by in situ hybridization with chromosome 7 (green) and 8 (red) centromere probes. Chromosome 7 and 8 in WBC of same patient are diploid. Blue color shows nucleus stained by DAPI. X 19500

5.   Absence or the presence of certain proteins and gene copy numbers such as in androgen receptors (see FIG.9), estrogen receptors (see FIG.10), progesterone receptors (see FIG.11), Vitamin D receptors, etc. (see FIG.12) can be analyzed, which provides valuable information for patient therapy design and management. Thus, information about the circulating cancer cells for 6-8 epithelial cancer obtained from this "Blood Biopsy" will provide valuable, previously unavailable data to the attending physicians of these patients.

FIG.9 FISH assay showing an increase in the number of gene copies of the androgen receptor gene of circulating prostate cells from a patient. Genes (red), cytokeratin (green), DAPI (blue).

FIG.11 Presence of progesterone receptors (red) in T47D breast cancer cells. Cytokeratin (green), DAPI (blue).

FIG.10 Presence of estrogen receptors (red) in MCF-7 breast cancer cells. DAPI (blue).
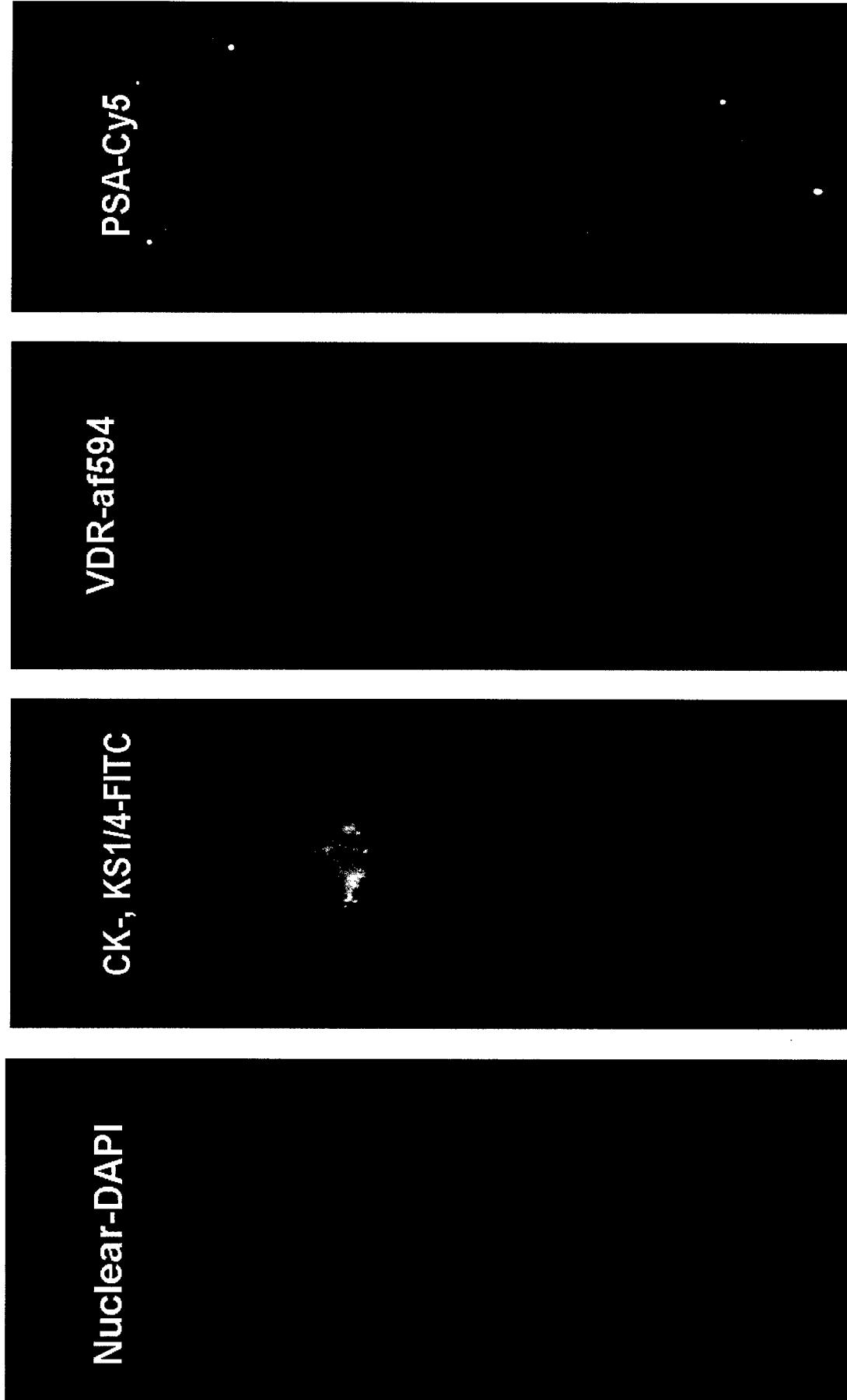
FIG.12 One prostate cancer cell (LnCAP) with WBC's, stained for nuclear DNA, epithelial cell characteristics (cytokeratins, KS ¼), Vitamin D receptor, and cellular PSA.

"Blood Biopsy" for Epithelial Cancer Cells Based on Circulating Cancer Cell Tests
AACR Conference
March 27, 2001

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In addition, the CCCT will provide very valuable information for basic oncology research about leakage of cancer cells from the primary tumors and the secondary tumors into circulation as well as the fate of these circulating cancer cells in the body. In a recent publication (Cancer, 88: 2787-2795, 2000), we provide evidence which strongly suggests that the circulating cancer cells can divide and multiply in the blood (see FIG.13 and FIG.14), and this information was not known before. These circulating microtumors can be the initial bridgehead for metastasis.
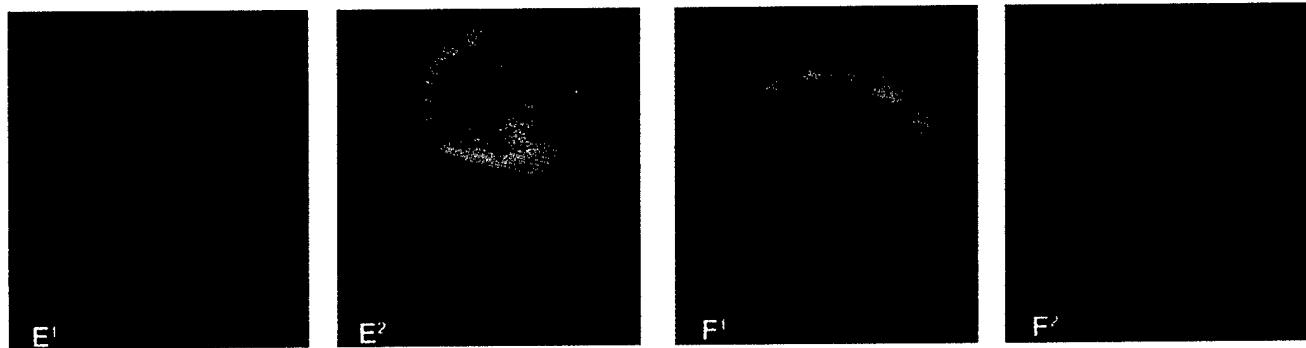

FIG.13 Circulating prostatic carcinoma cells were stained by using cytokeratin (CK) antibodies (green) and PSMA antibody or its mRNA probes (red), 4,6-diamidino-2-phenylindole (DAPI) for DNA nuclear staining (blue), for the detection of chromosomal centromeres or genes in the nucleus. (E^1 and E^2) A stem cell-like cancer cell is stained by CK (green) and PSMA mRNA (red) cytoplasmic probes. (F^1 and F^2) Two different stages of mitotic, dividing cancer cells have PSMA mRNA (red) and CK (green) positive staining.
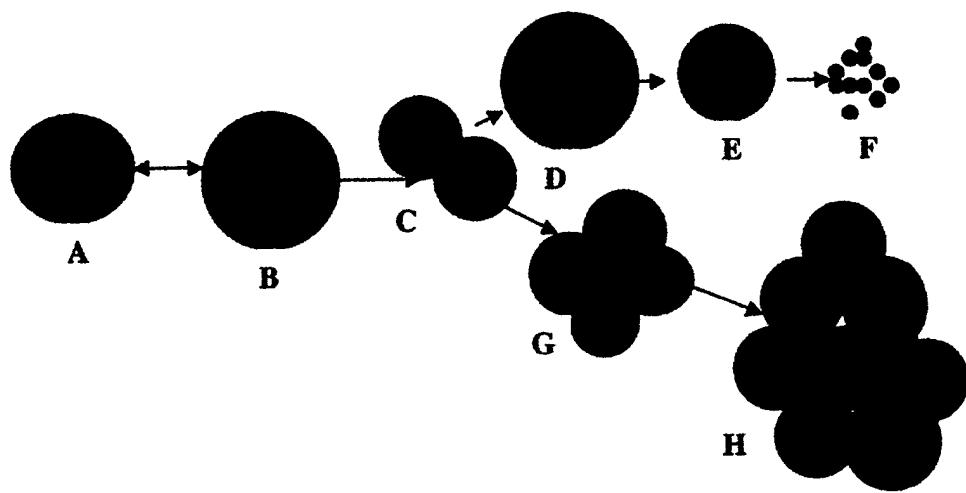

FIG.14 Cytologic dynamics of circulating cancer cells are demonstrated. The cells in this graph show a stem cell-like cancer cell (A), dividing cancer cells (B and C), a terminal cancer cell (D), cancer cellular derivatives (E and F), and circulating microtumors (G and H).

MARCH 28, 2001

Taking the Stealth out of Cancer's Spread

A powerful new test hones in on diseased cells in the bloodstream after the initial treatment. It could offer lifesaving alerts. As every cancer patient knows, you can't rest easy once the initial treatment is completed, no matter how successful it has been. It is devilishly hard to root out every last cancer cell, and any left behind could eventually start spreading again, often to a completely new site in the body via the bloodstream. It is this process of metastasis that most often proves fatal, rather than the initial tumor. A powerful new blood test, however, is proving remarkably accurate in measuring minute amounts of cancer cells in the blood, giving doctors a tool for determining very quickly how successful a therapy has been in eradicating the disease.

At the annual meeting of the American Association of Cancer Research in New Orleans on Mar. 27, Dr. Paul Ts'o of Johns Hopkins Medical Center revealed that a new measure called the Circulating Cancer Cell Test (CCCT) is able to detect and isolate as few as 10 to 20 cancer cells in 20 milliliters of blood. The technology, developed by Ts'o and licensed to Cell Works Inc. of Baltimore uses specially designed antibodies attached to fluorescent dyes to hone in on the cancer cells. The blood cells are then removed and the cancer cells studied with a powerful automated microscope.

Ts'o says the test has proved effective in identifying more than 30 different cancer cell lines, making it a universal test. Once the cancer cells are identified, they can then be analyzed to determine their structural makeup and biological activities -- important information for researchers seeking to develop highly targeted treatments for specific tumors.

Cell Works, which does all the blood analysis at its facility in Baltimore, will make the test generally available to doctors and researchers for prostate, breast, and colon cancer on Apr. 1, at a cost of $400 for the basic test.

By Cathy Arnst in New Orleans
Edited by Douglas Harbrecht

To Appear in the June 2001 issue of Oncology News International and Primary Care & Cancer .

CH140REV - 05/08/01 • Scheduled for JUNE 2001   Galley 1

Blood Test Detects Epithelial Cancer Cells

■ NEW ORLEANS—A test that detects epithelial cancer cells in circulating blood, and gives detailed information about their characteristics, was described in the late-breaking session of the 92nd Annual Meeting of the American Association for Cancer Research (AACR).

The Circulating Cancer Cell Test (BloodBiopsy) addresses the need for early detection of cancer recurrence, early diagnosis of metastasis, and early information about the response to therapy, according to principal investigator Paul O.P. Ts'o, PhD.

"From 20 mL of blood, we can get a definitive indication that a patient has cancer and that the cancer is in the circulation already. The consequences of this, however, we do not know yet," said Dr. Ts'o, professor of biochemistry, Johns Hopkins School of Hygiene and Public Health, and chairman and CEO of Cell Works Inc (Baltimore), developer of the test.

"We can find one cancer cell in 1 to 2 mL of blood or one cancer cell in 10 to 12 million white blood cells. The approach we take is to exclude all the blood cells and leave only the cancer cells behind," he said at a press conference.

The procedure utilizes double-gradient sedimentation for the removal of most red blood cells and white blood cells as well as magnetic cell sorting for the additional removal of white blood cells. The epithelial cells that remain are intact, without the attachment of magnetic beads or other foreign material, as may be the case with other positive-selection systems.

The cells are fixed, stained with various specific molecular probes, and scanned with an automated spectroscopic system.

The spectroscope captures a fluorescent digital image at low magnification, and further analyses are performed at higher resolution. Cancer cells and markers are identified on the basis of intensity and blob analysis (see Figure).

This test can routinely provide five types of basic information about individual cancer cells found in the blood sample, Dr. Ts'o said.

1. The test shows the number of cancer cells in the sample. The recovery rate of epithelial cells is 50% to 80%. Preliminary data on blood samples from 13 prostate cancer patients indicate that if there are 3,000 circulating cancer cells in the whole body, several cancer cells would be detected in 20 mL of blood.

2. The test analyzes the nuclear DNA content of the epithelial cells found in blood relative to the patient's white blood cells, reported as a DNA index. The epithelial cells have abnormally high DNA content compared to the normally diploid white blood cells on the same slide, and abnormally large two-dimensional nuclear area. These data confirm as neoplastic an observed epithelial cell with abnormal DNA content or nuclear size.

3. Levels of thymidylate synthetase and DNA fragmentation indicate growth status of the cell, and such analysis is performed on the same slide to identify and compare the growing cells vs the dying ones.

4. The test reveals information indicative of chromosomal abnormalities.

5. The test shows the absence or presence of certain proteins, such as androgen and estrogen receptors. This provides information useful in designing therapeutic strategies and patient management, Dr. Ts'o said.

"Now we are studying the uses of this information. This method should be useful especially during the 'silent period' between treatment of the primary tumor by surgery and radiation therapy and a recurrence," he said, and clinical studies are currently pursuing this aim.

The test should also reveal much about the leakage of cancer cells from the primary and secondary tumors, as well as the fate of these cells in the body. Studies have strongly suggested that the circulating cancer cells can divide and multiply

in the blood (*Cancer* 88:2787-2795, 2000)—information previously unknown, Dr. Ts'o said. "These circulating microtumors can be the initial bridge for metastasis," he commented.

In discussing this report at the press conference, Carol Prives, PhD, of Columbia University, said that the study "has great ramifications" and represents "a big advance" in early detection of occult cells.

"The test takes advantage not only of well known markers but also newer markers for changes in cancer cells," Dr. Prives said. "We know cancer cells will grow rapidly but at the same time will die rapidly. This test can assess DNA division as well as the DNA fragments that occur when cells start to die, so the technology is promising for being able to get microscopic dissections of circulating cancer cells in the blood."

Three Tests Available

Circulating Cancer Cell Tests are currently available from Cell Works for prostate, breast, and colon cancer, and the company is developing tests for other cancers, including gastric, liver, kidney, and lung cancer, and melanoma. The test is intended to be used on patients with known cancer etiology and is not a screening test. The company licenses the patented technology for the tests from Johns Hopkins University.

A medical laboratory permit issued by the Maryland Department of Health and Mental Hygiene and a certificate issued by the US Health Care Financing Administration allow Cell Work's state-licensed reference laboratory to accept samples from physicians in more than 40 states. For more information, consult the website www.cell-works.com. ∇

[caption for figure of cancer cells]

Prostate cancer cells (top), breast cancer cells (middle), and colon cancer cells (bottom) obtained and imaged using Cell Works' Circulating Cancer Cell Tests (BloodBiopsy).